Title: The balance between two isoforms of LEF-1 is a regulator in the growth of colon carcinoma

Authors:

Shu-Hong Wang (wsh2003@126.com)
Ke-Jun Nan (nankj@163.com)
Yao-Chun Wang (Tanking@sina.com)
Wen-Juan Wang (Wangwenjuan1983@tom.com)
Tao Tian (Tiantao0607@163.com)

Version: 4 Date: 6 January 2012

Author's response to reviews: see over
Dear Dr Timothy Shipley,

Re: Manuscript reference MS: 6024328605352270

Please find attached a revised version of our manuscript “The balance between two isoforms of LEF-1 is a regulator in the growth of colon carcinoma”, which we would like to resubmit for publication as an original article in *BMC Gastroenterology*.

Your comments and those of the reviewer were highly insightful and enabled us to greatly improve the quality of our manuscript. In accordance with the reviewer’s suggestion, we changed the title of our manuscript and modified Figures 7. In the following pages, please find our point-by-point responses to each of the comments of the reviewer as well as responses to your comments.

Revisions in the text are shown using red highlighting for additions, and strikethrough font highlights for deletions. We hope that the revisions in the manuscript and our accompanying responses will be sufficient to make our manuscript suitable for publication in *BMC Gastroenterology*.

We shall look forward to hearing from you at your earliest convenience.

Yours sincerely,

Dr Shu-Hong Wang PhD
Department of Medical Oncology
The First Affiliated Hospital of the School of Medicine of Xi'an Jiaotong University
Xi'an 710061, China
Tel: +86-29-85324086
Email: wsh2003@126.com
Responses to the Reviewer’s comments

Major comment:

1 Question: Cells are compared after analysis of the cell cycle by flow cytometry. Rather surprisingly, no increments in the sub G0/G1 population are detected for both the SW480-dL and HT29-dL cells. This would be expected based on the result shown in Figure 3 where an increase in Annexin-V positive cells is observed for both these cell lines. The authors need to provide an explanation for this discrepancy and preferably should include the percentage cells in sub G0/G1 in the analysis shown in Figure 2.
Response: We have re-analyzed the cell cycle by PI staining and the apoptosis by Annexin-V staining for three more times. No significant peaks of the sub G0/G1 population were detected for both the SW480-dL and HT29-dL cells. But the apoptosis of these cells can be detected by Annexin-V staining. We think that there are two main reasons as follow: (1) As we known, Annexin-V, belonging to the protein family of annexins, with anticoagulant properties has proven to be a useful tool in detecting early apoptotic cells since it preferentially binds to negatively charged phospholipids like PS in the presence of Ca$^{2+}$ and shows minimal binding to phosphatidylcholine and sphingomyeline. Changes in PS asymmetry, which is analyzed by measuring Annexin-V binding to the cell membrane, were detected before morphological changes associated with apoptosis have occurred and before membrane integrity has been lost. Apoptotic cells become Annexin-V positive after nuclear condensation has started, but before the cell becomes permeable to PI. Therefore, as tools of detection for apoptosis, Annexin-V staining is more sensitive than DNA flow cytometric analysis. In our results, the cells we detected may be in the stage of early apoptosis, which could be detected by Annexin-V staining but not by cell cycle analysis. (2) SW480 and HT29 cells are adherent, which should be disaggregated to have a single cell suspension before Annexin-V staining. Any procedure which affects the integrity of the plasma membrane will result in cell positive for Annexin-V. The binding of Annexin-V to phosphatidyserine may be affected in adherent cells, which are usually detached from plastic dishes by enzymatic treatment, although their membrane integrity is not altered. So Apoptosis-positive rate of HT29-dL cells detected by Annexin-V staining would be higher than that detected by cell cycle analysis.

For the above reasons, Annexin-V positive cells in our experiment could not form significant peaks of the sub G0/G1 population in cell cycle analysis.

2 Question: The authors should evaluate whether reduced tumor vascularization observed for cells expressing the LEF-1-dL variant is also significantly reduced in tumors of the same size. However, in Figure 6A the authors show and claim that tumors were of different sizes after 15 days.
Incidentally, this statement also contrasts with data shown in panel 6C where tumors of appreciable volumen were not really detectable until after 20 days.

**Response:** Thank reviewer’s careful reading, which help us find the error ignored in **Figure6 legend**. As what we have said in **Tumor formation** of **Methods**, tumor growth was monitored every 3 days by measuring the tumor length (L) and width (W) with a sliding caliper from 18 days after the initial inoculation. 30 days after the initial inoculation, tumors were excised, weighed and examined for vascularization. This error has been corrected in the legends of **Figure6 and Figure7**.

According to the suggestion of reviewer, microvessels in colon tumors produced by HT29-LEF-1-ρL and controls in nude mice were further detected by immunohistochemistry for CD31, VEGFR2 and HIF1α on 18th day after the initial inoculation. As shown in **Figure6C**, HT29 tumors in different groups were approximately the same size (about 50mm^3^) on 18th day. Tumor vascularization of HT29-LEF-1-ρL was reduced slightly compared with the controls shown in **Figure 7E, G and F**, but no statistical differences have been got (CD31: P=0.292; VEGFR2: P=0.521; HIF1α: P=0.643). Therefore, reduction in tumor vascularization of HT29-LEF-1-ρL formed gradually during the process of tumor growth, rather than in the initial stages of tumor formation.

**3 Question:** The authors have changed the title. However, no evidence is provided indicating that LEF-1 variants are “key” regulators. Changes in many molecules will induce similar changes.

**Response:** The title “Truncated LEF-1 is one key regulator in the growth of colon carcinoma” has been changed to “The balance between two forms of LEF-1 is a regulator in the growth of colon carcinoma”.